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AIB1 (Amplified in Breast Cancer 1) is a nuclear receptor coactivator found amplified and overexpressed in a subset of breast cancers. AIB1 potentiates the transcriptional activity of the estrogen, progesterone, thyroid hormone, and retinoid receptors. It is necessary for normal female reproductive function and mammary gland development and is rate-limiting for tumor growth in nude mice. It is hypothesized that the amplification and overexpression of AIB1 contribute to the promotion and/or progression of breast cancer. An understanding of the regulation of expression of AIB1 will give insight into the role of AIB1 in breast cancer. This work has shown that antiestrogens, retinoids and TGF-β contribute to the overexpression of AIB1 in MCF-7 breast cancer cells and will identify specific positive and negative regulatory elements of the AIB1 promoter that contribute to expression of AIB1. Further studies will explore the consequences of overexpression of AIB1 through stable transfection into a cell line with low endogenous levels of AIB1.

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INTRODUCTION

AIB1 (**A**mplified **i**n **B**reast cancer **1**) is a nuclear receptor coactivator found amplified and overexpressed in a subset of breast cancer (1, 2). Gene amplification also correlates with estrogen and progesterone receptor positivity of primary breast tumors, as well as with tumor size (2). As a coactivator, AIB1 is able to potentiate the transcriptional activity of the estrogen, progesterone, thyroid hormone, and retinoic acid receptors *in vitro* (1, 3). Disruption of the AIB1 gene results in reduced female reproductive function and blunted mammary gland development in mice (4). Targeted AIB1 reduction in breast cancer cells reduced estrogen-dependent colony formation is soft agar and tumor growth in nude mice (5). Based on these findings, AIB1 is an important factor in tumorigenesis. An understanding of the regulation of expression of AIB1 will give insight into the role of AIB1 in cancer. Specifically, the research done with the funding of this grant will identify and examine the regulatory pathways resulting in changes in AIB1 expression levels in response to growth factors, hormones/hormone antagonists, and other relevant agents.

BODY

Task 1: To identify the factors and mechanisms resulting in increased AIB1 mRNA levels. (Months 1-24) **See appended manuscript for figures.**

    a. Analyze the expression levels of AIB1 mRNA in response to hormones, hormone antagonists and other possibly relevant growth factors by Northern blot analysis. (Months 1-6)

MCF-7 breast cancer cells were treated with estrogen, the antiestrogens ICI 182,780 (ICI) and 4-hydroxytamoxifen (OHT), and all-*trans* retinoic acid (RA) over a period of 48 hours and analyzed for AIB1 mRNA expression by Northern blot. AIB1 mRNA was found to be upregulated 2-4 fold in response to ICI, OHT, and RA, and unchanged in the presence of estrogen (Figures 2-4). Further analysis demonstrated that removal of estrogen, by growth in 1% CCS, resulted in an upregulation of AIB1 mRNA and a subsequent addition of estrogen was then able to reverse this induction (Figure 1). AIB1 protein expression levels were also affected in the same manner following Western blot analysis (Figures 3,4).

    b. Perform ChIP assays and others to analyze the mechanisms resulting in AIB1 overexpression in response to hormone/hormone antagonists. (Months 7-12)

To address the mechanism by which AIB1 mRNA is upregulated in response to ICI and RA, I performed Northern blot analyses using actinomycin D (actD), which blocks transcription, in the presence of estrogen, ICI, and RA (Figure 5). The transcription half-

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life of AIB1 was unaffected by any of these agents, suggesting that the increase in AIB1 mRNA levels is the result of increased transcriptional activity rather than stabilization of the AIB1 transcript. This was further supported by the results of a chromatin immunoprecipitation (ChIP) assay that showed an increase in RNA Pol II on the AIB1 gene following treatment with ICI (Figure 6).

I then performed further Northern blot analyses to determine whether the increased expression of AIB1 mRNA by ICI and RA was the result of induction of an intermediary protein that regulated AIB1 expression. Treating MCF-7 cells with cycloheximide, to block *de novo* protein synthesis, in the presence or absence of ICI or RA resulted in a moderate repression of the induction by these agents, indicating that at least part of the response to ICI and RA occurs through a secondary protein (Figure 7).

A review of the scientific literature revealed that both ICI and RA are able to induce expression and secretion of TGF-β (6). Northern blot analysis of MCF-7 cells in the presence of TGF-β resulted in an increase in AIB1 mRNA (Figure 8a). To then determine if TGF-β is the protein through which ICI and RA are able to increase AIB1, a blocking antibody to TGF-β was used in the presence of ICI or RA. This TGF-β blocking antibody was able to partially repress the induction of AIB1 mRNA by both ICI and RA, indicating that a portion of the upregulation is the result of TGF-β activity in response to ICI and RA (Figure 8b).

    c.   Obtain/clone the AIB1 gene promoter and begin analysis of regulatory elements. (Months 13-24).

I have verified by primer extension the 5' end of the AIB1 transcript. With the publication of the sequences from the human genome project, I was able to identify the first exon of AIB1 in the genomic sequence and identified the upstream sequences as the putative promoter for AIB1. I obtained this sequence in BAC clone RP11-456N23 from the Sanger Centre. I designed PCR primers and amplified a portion of genomic sequence that included ~50 bp of exon 1 of the AIB1 gene and 1.5kb of upstream sequence. This sequence has been cloned into the pGL3 basic luciferase reporter vector (Promega) and studies are ongoing to determine the activity of this putative promoter in response to estrogen, ICI, and RA.

Task 2: Development and phenotypic characterization of cell lines that stably overexpress AIB1. (Months 25-36)

    a. Transfect breast cancer cell lines with AIB1 and pick clones expressing the highest levels of AIB1 by Northern and Western blot analysis. (Months 25-30)

    b. Analyze the overexpressing cell lines for changes in rates of proliferation and anchorage independent growth. (Months 31-36)

These tasks have not yet been addressed. I currently have two cell lines that express moderate (T47D breast cancer cells) and very low (MCF-10A breast epithelial cells) endogenous levels of AIB1 as candidates for stable transfection of AIB with the pCMX-ACTR A38 vector.

KEY RESEARCH ACCOMPLISHMENTS

- Identification of 3 key agents, ICI, RA, and TGF-β, that can upregulate AIB1 expression.

- Partial elucidation of the mechanism through which these agents act to upregulate AIB1 expression.

- Identification of the putative AIB1 promoter.

REPORTABLE OUTCOMES

"TGF-β mediates estrogen and retinoid regulation of the nuclear receptor coactivator AIB1 in MCF-7 breast cancer cells." **Kristina J. Lauritsen**, Heinz Joachim List, Ronald Reiter, Anton Wellstein, and Anna T. Riegel

- Accepted by *Oncogene* June 2002, pending revision

CONCLUSIONS

To date there is limited information on the hormonal or growth factor regulation and expression of the steroid receptor coactivator genes. We have shown, for the first time, the regulation of AIB1 in a human breast cancer cell line, MCF-7. We show that estrogen is able to repress AIB1 gene expression and that AIB1 expression is upregulated following treatment with ICI, OHT, RA, and TGF-β. A putative promoter for AIB1 has been identified, cloned, and is undergoing analysis to determine specific positive and negative regulatory elements in the promoter that affect AIB1 expression.

Because AIB1 has been shown to be amplified and overexpressed both in breast tumor tissues and cell lines, as well as rate-limiting in hormone-dependent breast tumor growth, the regulation of AIB1 expression in breast cancer cell lines by antiestrogens and RA has many implications. Suppression of AIB1 by estrogen may be part of a normal reaction to hormone, as a way to dampen or attenuate the response to steroidal stimulation. This normal control may be subverted during antiestrogen therapy, sensitizing the cells to ER or other proliferative signals e.g. through PR or other growth factor stimulated pathways (7). These pathways would not be blocked by antiestrogen therapy and may provoke a tamoxifen/antiestrogen resistant phenotype. Another important consideration is the uterine stimulatory effect of tamoxifen treatment of breast cancer that has been associated with an increased incidence of endometrial cancer. Whether tamoxifen is able to stimulate AIB1 expression in endometrial cells has yet to be shown, but may be an important secondary consideration in breast cancer therapy.

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TGF-β mediates estrogen and retinoid regulation of the nuclear receptor coactivator AIB1 in MCF-7 breast cancer cells

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Running Title: Estrogen and retinoid regulation of AIB1.

Abstract

AIB1 (amplified in breast cancer 1) is a nuclear receptor coactivator gene amplified and overexpressed in breast cancer. However, the mechanisms by which AIB1 is regulated are unclear. Here we show that 17β-estradiol represses AIB1 mRNA and protein expression in MCF-7 human breast cancer cells primarily by suppressing AIB1 gene transcription. Estrogen levels present in fetal calf serum are sufficient to maintain AIB1 mRNA and protein at low basal levels, and this repression is reversed by the addition of antiestrogens or all-*trans* retinoic acid. Interestingly, cycloheximide inhibition experiments revealed that secondary protein synthesis was necessary to induce AIB1 expression by antiestrogens and retinoids. Experiments with TGF-β and TGF-β blocking antibodies demonstrated that this growth factor modulates AIB1 expression and showed that the antiestrogen and retinoid induction of AIB1 gene expression is mediated predominantly through TGF-β. These data reveal a mechanism of estrogen-induced down-modulation of the overall hormone sensitivity of cells through feedback inhibition of coactivator gene expression. These data also suggest that antiestrogens can shift the sensitivity of cells to non-estrogenic proliferative signaling by increasing cellular levels of AIB. This effect may play a role in breast cancer progression and resistance to drug treatment.

Introduction

Nuclear hormone receptors are ligand-dependent transcription factors that regulate the expression of genes critical to biological processes such as proliferation, differentiation, development, reproduction, and homeostasis. Recent discoveries have shown that these receptors are often associated with specific cofactors, corepressors and coactivators, that influence their transcriptional activity (McKenna et al., 1999; McKenna & O'Malley, 2002). In the absence of ligand, some of the nuclear receptors are bound to corepressors such as SMRT and NCoR. After ligand binding, the corepressors are released and nuclear receptor coactivators are recruited. The interaction of the nuclear receptor with its coactivator then leads to enhancement of the transcriptional activity of the nuclear receptor. Some of the best-characterized nuclear receptor coactivators belong to the p160/SRC-family. This family consists of SRC-1, TIF-2/GRIP1 and AIB1/ACTR/RAC3/TRAM-1/SRC-3 (Anzick et al., 1997; Chen et al., 1997a; Guan et al., 1996; Li et al., 1997; Onate et al., 1995; Suen et al., 1998; Takeshita et al., 1997; Voegel et al., 1996; Walfish et al., 1997).

Interestingly, one of these coactivators, AIB1, was found amplified in breast, ovarian, pancreatic, and gastric cancer (Anzick et al., 1997; Ghadimi et al., 1999; Guan et al., 1996; Sakakura et al., 2000). Amplification of the AIB1 gene was correlated with estrogen and progesterone receptor positivity of primary breast tumors as well as with tumor size (Bautista et al., 1998). Independent of any amplification event, increased expression levels of AIB1 also have been found in primary breast tumors (Bouras et al., 2001; Guan et al., 1996; List et al., 2001b). AIB1 enhances the transcriptional activity of

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the estrogen, progesterone, thyroid hormone, and retinoic acid receptors *in vitro*. (Anzick et al., 1997; Chen et al., 1997a; Guan et al., 1996; Li et al., 1997). Cotransfection studies with AIB1 and p53 have shown that AIB1 inhibits p53-dependent transactivation (Lee et al., 1999a). Taken together, these findings led to the hypothesis that AIB1 contributes to the development of breast cancer. Furthermore, a recent study demonstrated that disruption of the AIB1 (SRC-3, p/CIP) gene in mice results in a pleiotrophic phenotype where mice display dwarfism, delayed puberty, reduced female reproductive function and blunted mammary gland development (Wang et al., 2000; Xu et al., 2000). AIB1 also seems to play a role in the growth hormone regulatory pathway, as well as in the production of estrogen in mouse development (Wang et al., 2000; Xu et al., 2000). We have recently shown that AIB1 exerts a rate-limiting role in hormone-dependent MCF-7 human breast cancer cells (List et al., 2001a). In these studies, reduction of endogenous AIB1, through ribozyme targeting, reduced estrogen-dependent colony formation in soft agar and tumor growth in nude mice (List et al., 2001a).

Based on these findings we were interested in the regulation of AIB1 expression levels by antiestrogens and retinoids, agents that are currently used or tested for breast cancer therapy. In this study, we determined that estrogen can suppress AIB1 gene expression and that antiestrogens and retinoids can reverse this repression. TGF-β can also induce AIB1 gene expression and antibody-blocking experiments revealed that a significant portion of the antiestrogen and retinoid induction of AIB1 mRNA was through secondary induction of TGF-β secretion. The opposing regulation of AIB1 gene

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expression by estrogen, antiestrogens, retinoids, and TGF-β has important implications

for breast cancer development and therapy.

Results

Effect of estrogen and antiestrogens on AIB1 gene expression

We observed in a number of experiments with MCF-7 breast cancer cells that serum withdrawal resulted in a significant upregulation of AIB1 mRNA levels. Serum contains sufficient estrogen to produce maximal estrogen responses and these can be blocked by antiestrogens (Lippman & Bolan, 1975). To determine if estrogens directly suppress AIB1 mRNA we kept cells for 48 h in charcoal stripped serum and observed that addition of 17β-estradiol reduced AIB1 mRNA levels, with a significant reduction being observed as early as 4 hrs after the initiation of treatment (Figure 1). In fact, AIB1 mRNA levels were reduced to basal levels (Figure 1) after 24 hrs exposure to estrogen.

To further investigate if the suppressive effect of serum was through estrogens, we treated cells grown in full serum with additional estrogen and found that no further suppression of the basal levels of AIB1 mRNA in the MCF-7 cells was observed (Figure 2a). This suggests that the estrogen levels in serum are sufficient for full suppression of AIB1 mRNA levels. Consistent with this effect being mediated by estrogens, treatment with the steroidal antiestrogen ICI 182,780 (ICI) resulted in a rapid 2.5-fold induction of AIB1 mRNA levels (Figure 2b). The non-steroidal antiestrogen 4-hydroxytamoxifen (OHT) induced a slightly less than 2-fold increase in AIB1 mRNA expression (Figure 2c). Finally, we confirmed that induction of AIB1 mRNA by serum withdrawal was the same as antiestrogen treatment since both can be effectively reversed by subsequent treatment with 17β-estradiol (compare Figure 1 vs. Figure 2d).

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The estrogen-mediated repression of AIB1 expression at the mRNA level is paralleled by a reduction in AIB1 protein (Figure 3a). Consistent with this, AIB1 protein is increased by 2-fold after antiestrogen treatment of MCF-7 cells (Figure 3b). Interestingly, the hormone and antagonist effects were observed for up to 72 hrs following treatment. This suggests that sustained changes in AIB1 protein levels are induced after treatment with estrogen or antiestrogen and are not transitory adaptations of the cells to a new stimuli.

*Induction of AIB1 gene expression by all-*trans *retinoic acid*

To test if the AIB1 gene is responsive to any other compounds used in the hormonal therapy of breast cancer, we also tested the effect of all-*trans* retinoic acid (atRA), which in some assays antagonizes estrogen effects (Segars et al., 1993). Furthermore, a previous study has shown that atRA is able to induce expression of AIB1 mRNA in another cancer cell line, HL-60 cells, a promyelocytic leukemia cell line (Li & Chen, 1998). We treated MCF-7 cells with atRA in serum-free media for different time periods and found an induction of AIB1 mRNA expression of up to 3.5-fold that begins as early as 6 h after treatment and is maintained for at least 48 h (Figure 4a). Western blot analysis of cells treated with atRA demonstrates an increase of the AIB1 protein (5-fold) that is maintained for at least 72 h after treatment (Figure 4b).

The preceding data suggests that atRA reverses the effect of estrogen suppression and we next wanted to see if this atRA induction, similar to antiestrogens, could be reversed by the addition of estradiol. A similar response to the estradiol-mediated

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repression of the ICI induction of AIB1 mRNA was seen following the induction of AIB1 mRNA by atRA (Figure 4c). In these experiments, estradiol was able to significantly reverse the induction as early as 4 h after treatment, and only slightly more at later time points to levels no longer significantly different from basal.

AIB1 Regulation is Independent of Cell Cycle Regulation

Because antiestrogens and atRA inhibit and estradiol stimulates proliferation of MCF-7 breast cancer cells, we explored the possibility that the induction of AIB1 mRNA in response to these agents might coincide with effects on the cell cycle (Table 1). Examination of the percent of cells in the S phase versus the G0/G1 phase shows that following 6 h of treatment with each respective agent, a timepoint at which we detected an increase in AIB1 mRNA levels, there is no concomitant change in the cell cycle status of those cells. Therefore, while the highest levels of AIB1 mRNA may correlate with the lowest %S and highest %G0/G1, the increased levels of AIB1 during the first 24 h of treatment with each respective agent do not coincide with a decrease in %S or an increase in %G0/G1. Thus, we conclude that the effects of these ligands are directly on AIB1 gene expression rather than indirectly because of proliferative events in the cell.

Half-Life of AIB1 mRNA is not affected by treatment with 17β-estradiol, ICI, or atRA

Treatment of MCF-7 cells in serum free-media in the presence of the transcriptional inhibitor actinomycin D shows an AIB1 mRNA half-life of approximately 4-6 h (Figure 5). There are no significant changes in the half-life of the AIB1 mRNA upon the addition of estradiol, ICI, or atRA indicating that the sustained high levels of AIB1 mRNA in

response to ICI and atRA (Figures 2 and 4) are due to an increase of AIB1 transcription rather than a stabilization of the transcript.

Antiestrogen induced changes in AIB1 mRNA occur at the transcriptional level.

To determine if the increase in AIB1 mRNA is a consequence of direct stimulation of transcription, a chromatin immunoprecipitation (ChIP) assay was performed using an antibody to RNA Pol II to pull down genes actively undergoing transcription. This allowed us to assay the relative amounts of RNA Pol II engaged on the AIB1 gene in the ICI-treated versus untreated MCF-7 cells. MCF-7 cells were subjected to the AIB1 suppressive effects of estradiol in serum-free media for 48 h followed by 2 hours of ICI treatment in serum-free media. The result demonstrates a significant 4-fold increase in transcription of the AIB1 gene following ICI stimulation while cells treated with estrogen had no detectable transcription over background levels when compared with the no antibody control (Figure 6). The lack of effect of estrogen on transcription was expected since the cells had been pretreated for 48 h in serum containing estrogen and basal transcription of AIB1 would be very low under these conditions. While this data clearly demonstrates that the antiestrogen stimulates an increase in transcription, it does not resolve the question of the mechanism of induction, whether by a direct effect of an antiestrogen-receptor complex on the AIB1 promoter or through induction of a secondary factor that stimulates AIB1 mRNA synthesis.

Antiestrogen and retinoid upregulation of AIB1 mRNA is mediated through TGF-β

In order to determine a possible mechanism for the upregulation of AIB1 we studied whether transcriptional stimulation by ICI and atRA requires protein synthesis to stimulate transcription. Treatment of MCF-7 cells with the protein synthesis inhibitor cycloheximide (CHX) in the presence and absence of ICI or atRA demonstrates that protein synthesis is necessary for the induction of AIB1 transcription by ICI and atRA (Figure 7). A possible candidate protein that might mediate this effect is TGF-β since antiestrogens have been shown to directly increase translation and secretion of TGF-β in MCF-7 cells (Knabbe et al., 1987). Interestingly, in the A549 lung carcinoma cell line, TGF-β was able to induce an increase in AIB1 levels (Akiyama et al., 2000). In addition, other studies support a role for atRA in the production of TGF-β (Choudhury et al., 2000; Degitz et al., 1998; Jakowlew et al., 2000). Therefore, we postulated that if TGF-β is able to effect an increase in AIB1 mRNA in MCF-7 cells, then the induction by ICI, OHT and atRA might be a result of stimulation of TGF-β, which in turn induces AIB1 mRNA. To examine this, we first treated MCF-7 cells with a single dose of TGF-β and found that it is able to increase AIB1 mRNA levels 2-fold 12 h after treatment, with mRNA levels returning to basal values 48 hours after treatment (Figure 8a). Because TGF-β was able to produce an induction of AIB1 mRNA levels, we next tested the hypothesis that the induction by ICI and atRA is mediated through a TGF-β increase. For this, we treated MCF-7 cells for 24 h with ICI and atRA with or without a TGF-β blocking antibody. The blocking antibody prevented most of the induction of AIB1 mRNA by both ICI and atRA, demonstrating that TGF-β contributes significantly to the induction of AIB1 mRNA by ICI and atRA (Figure 8b).

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Discussion

To date there is limited information on the hormonal or growth factor regulation and expression of the steroid receptor coactivator genes. SRC-1, the first member of the SRC family, was shown to be upregulated in GH3 rat pituitary cells by thyroid hormone, as well as a small *in vivo* induction in the rat pituitary gland (Misiti et al., 1998). Conversely SRC-1 mRNA was downregulated in GH3 cells and in *in vivo* studies by the administration of estradiol (Misiti et al., 1998). AIB1 (RAC3) has been previously described to be regulated by atRA in HL60 and NB4 promyelocytic leukemia cell lines resulting in significant enhancement of AIB1 mRNA expression (Li & Chen, 1998). Using gene trapping in A549 human lung carcinoma cells AIB1 was described as one of a number of TGF-β responsive genes in these cells showing a small upregulation of expression (Akiyama et al., 2000). Alternately, *in vivo* studies of rat uteri following treatment with estradiol and OHT showed no alteration of expression of any coactivators of the SRC family, including AIB1 (Nephew et al., 2000). In the present study we show, for the first time, regulation of AIB1 mRNA expression in a human breast cancer cell line, MCF-7. We show that estrogens can suppress AIB1 gene expression, and report an upregulation of AIB1 following treatment with the antiestrogens ICI and OHT, as well as with atRA and TGF-β. In addition we demonstrate that estradiol present in serum is sufficient to repress the basal levels of AIB1 mRNA and that this suppression can be reversed by ICI and atRA. We propose that this feedback loop is one physiological mechanism whereby cells modulate the overall estrogenic signaling by regulation of a coactivator that is required for hormone regulation of a number of cellular genes.

AIB1 has been shown to be amplified and overexpressed in both breast tumor tissue and cell lines, and is also rate-limiting in hormone-dependent breast tumor growth. We have now shown, in the breast cancer cell line MCF-7, that the expression of AIB1 mRNA can be hormonally regulated. AIB1 has coactivator functions in conjunction with nuclear receptors, the TEF family of transcription factors (Belandia & Parker, 2000), as well as with p53 (Lee et al., 1999b) that are only now being elucidated. As such, the regulation of AIB1 expression in breast cancer cells by antiestrogens and atRA has many implications. The suppression of AIB1 by estrogen may be a normal reaction to hormone stimulation, a way of dampening or attenuating the response to steroidal stimulation and this normal control may be subverted during antiestrogen therapy. In its function as a coactivator of the estrogen receptor, upregulation of AIB1 may serve to increase sensitivity to low levels of ER and pave the way for eventual tamoxifen resistance seen in many breast cancers. Increasing AIB1 levels would either making the ER over-sensitive to stimulation or would sensitize the cell to other proliferative signals e.g. through PR or growth factor stimulated pathways (Reiter et al., 2001, Xu, 2000, 20300909). These pathways would not be blocked by antiestrogen therapy.

Conversely, in its role as a coactivator of other nuclear receptors, upregulation of AIB1 by antiestrogens, atRA, or through TGF-β may serve as a cellular defense mechanism to the antiproliferative properties of these agents by enhancing the transcriptional activity of nuclear receptors. Another important consideration is that the uterine stimulatory effect of tamoxifen treatment in breast cancer has been associated with an increased incidence of endometrial cancer. Whether tamoxifen is able to

stimulate an increase in AIB1 in human endometrial cells has yet to be shown, but may be an important secondary consideration in breast cancer therapy. Interestingly, because AIB1 is known to coactivate the RAR and RXR (Chen et al., 1997b), this upregulation is suggestive of an autoregulatory event where, in contrast to the ER negative feedback, AIB1 is continuously transcribed to ensure continuous transactivation by RAR and RXR. In both these systems it appears that AIB1 levels would be an important set point determinant of the extent of signaling through estrogens or retinoids.

Materials and Methods

Cell culture and reagents

The human breast cancer cell line MCF-7 was cultured in modified improved minimal essential medium (IMEM) containing L-glutamine (Life Technologies, Inc.) with 10% fetal bovine serum (Life Technologies, Inc.). ICI 182,780 (ICI), 17ß-estradiol, and 4-hydroxytamoxifen (OHT) were dissolved in ethanol, all-trans retinoic (atRA) acid was dissolved in dimethyl sulfoxide (DMSO). Recombinant human transforming growth factor β-1 (TGF-β-1) was obtained from Life Technologies, Inc. and a monoclonal antibody to TGF-β-1,2,3 was obtained from R&D Systems.

Cytoplasmic RNA preparation

MCF-7 cells were grown for 24 h in 15 cm dishes to 75% confluence in IMEM containing 10% fetal calf serum. Following this, cells were washed twice in serum-free IMEM, and treated with the indicated drug in serum-free IMEM. Cells, treated with ICI (10^{-8} M) or atRA (10^{-6} M) for 24 h before treatment with estradiol were washed twice in serum-free IMEM before estradiol treatment. Cytoplasmic RNA was isolated by scraping and collecting the cells in cold 1X PBS. Lysis buffer (0.2 M Tris-HCl, pH 8.0, 0.14 M NaCl, 2 mM $MgCl_2$, 0.5% Nonidet P-40) was added to resuspend pelleted cells on ice for 4 min. Nuclei were pelleted by a 15 sec spin (14,000 x g) and the supernatant transferred to a tube containing 0.5 ml phenol:chloroform:isoamyl alcohol (25:24:1 wt/vol) (PCI) (Life Technologies, Inc.) and 0.5 ml STE (5 mM Tris-HCl, pH 8.5, 2 mM EDTA, 0.2% SDS). Samples were vortexed and spun (14,000 x g) in an eppendorf centrifuge for 15 min at room temperature. The aqueous layer was removed to a new tube containing 0.5

ml PCI and the extraction was repeated. RNA was precipitated overnight at –20°C in an equal volume of ethanol.

Northern blot analysis

15 μg of cytoplasmic RNA were separated by electrophoresis in a 1.2% formaldehyde-agarose gel. The gel was soaked for 5 min in 0.1N NaOH, for 30 min in 20X SSC (0.9M sodium chloride, 0.09M sodium citrate, pH 7.0) and then blotted onto a nylon transfer membrane (Osmonics, Inc.). The blots were prehybridized in 6X SSC, 0.5% (wt/vol) SDS, 5X Denhardt's solution (0.1% (wt/vol) Ficoll, 0.1% (wt/vol) polyvinylpyrrolidone, 0.1% (wt/vol) bovine serum albumin, 100 μg/ml sonicated salmon sperm DNA) (Life Technologies, Inc.) for 4 h at 42°C. Hybridization was carried out overnight at 42°C in the same buffer. After hybridization, blots were washed three times with 2X SSC/0.1% SDS for 5 min at 42°C, one time with 1X SSC/0.1% SDS for 10 min at 42°C, and with 0.1X SSC/0.1% SDS for 10 min at 42°C. Hybridization probes were prepared by random-primed DNA labeling (Amersham Pharmacia Biotech) of AIB1 and human GAPDH (Clontech). The AIB1 probe is a 7.5 kb *Eco* RI-fragment from pCMX-ACTR A38, encompassing amino acids 703-927 of AIB1 (Chen et al., 1997a). Quantitation of mRNA levels was performed using a PhosphorImager (Molecular Dynamics).

Cell Cycle Analysis

MCF-7 cells were grown for 24 h in 10 cm dishes to 75% confluence, washed twice in serum-free IMEM, and treated with the indicated drug in serum-free IMEM. Cells were harvested using trypsin (Life Technologies) and verified microscopically to be in a single

cell suspension. The cell number was adjusted to 1-2 x 10^6 cells per tube and centrifuged (1000 x g) for 5 min. The supernatant was removed and cells were resuspended in 100 µl of citrate/DMSO buffer and transferred to polystyrene tubes (Falcon). Cells were frozen immediately in dry ice and stored at −80°C. Cell cycle analysis was performed by Vindelov staining using a flow cytometer (Vindelov et al., 1983).

Western blot analysis

MCF-7 cells were grown for 24 h in 10 cm dishes to 75% confluence, washed twice in serum-free IMEM, and treated with the indicated drug in serum-free IMEM. Cells were washed with 1X PBS, harvested with a cell scraper, and washed twice with wash buffer (10 mM HEPES, pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, protease inhibitor mixture (Complete™, Roche Molecular Biochemicals)). The cell pellet was resuspended in lysis buffer (20 mM HEPES, pH7.8, 1.5 mM $MgCl_2$, 420 mM NaCl, 25% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, protease inhibitor mixture, 0.1% Nonidet P-40) and incubated for 10 min on ice. The suspension was centrifuged at 14,000 x g at 4°C for 10 min. 40 µg of supernatant protein were electrophoresed on a 4-20% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane, and the membrane was incubated for 1 h at room temperature with 5% nonfat milk in PBST (PBS, 0.5% Tween 20) followed by washing four times for 5 min each with PBST. The membrane was incubated for 1 h at room temperature with primary anti-AIB1 antibody (Transduction Laboratories), washed as described above, and incubated for 1 h with a secondary antibody-peroxidase conjugate (10,000-fold dilution in PBST). After washing, the membranes were incubated for 1 min with ECL detection

solution (Amersham Pharmacia Biotech) and then exposed to film. Bands were quantitated using densitometry.

Formaldehyde Crosslinking and Chromatin Immunoprecipitation (ChIP Assay)

MCF-7 cells were grown in 15 cm dishes to approximately 50% confluence, washed two times with serum-free IMEM and treated for 48 h with 10^{-7} M estradiol in IMEM. Cells were then washed two times with serum-free IMEM and treated for 2 h with 10^{-8} M ICI 182,780. Proteins were crosslinked to DNA by adding formaldehyde directly to the culture medium to a final concentration of 1%. Cells were incubated 15 minutes at room temperature, washed, and scraped into 1 ml 1X PBS containing 1X protease inhibitor cocktail (Complete™; Roche Molecular Biochemicals). Cell pellets were lysed in 200 μl lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0, 1X protease inhibitor cocktail) for 10 min on ice. Lysates were sonicated on ice to an average length of 200 to 600 bp and centrifuged to remove cell debris. Supernatant was diluted 5-fold in immunoprecipitation buffer (0.1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate, 140 mM NaCl, 1X protease inhibitors) and pre-cleared with 50 μL GammaBind™ Plus Sepharose™ (Amersham Pharmacia Biotech), 20 μg salmon sperm DNA, and 50 μg BSA for 30 min at 4°C. Beads were pelleted and 10 μg of antibody was added to supernatant and incubated overnight at 4°C. Immune complexes were collected with GammaBind™ Plus Sepharose™ and washed (Orlando et al., 1997). DNA was eluted with 1% SDS, 0.1 M NaHCO₃ for 15 minutes at room temperature. Crosslinks were reversed by incubating eluates at 65°C for 4 hours in 0.2 M NaCl, followed by digestion with proteinase K (20 μg) in 10 mM EDTA, 40 mM Tris-HCl, pH 6.5 for 2 hours at

45°C. DNA was recovered by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. Specific AIB1 DNA was quantitated by real time PCR using the iCycler iQ Detection System (BioRad, Hercules, CA). Amplification was performed in a 25 µl final volume containing 1X reaction buffer (SYBR Green PCR core reagent kit, Applied Biosystems, Foster City, CA) 3 mM MgCl$_2$, 0.25 µl Platinum Taq polymerase (Life Technologies, Carlsbad, CA), 0.2 mM dNTP, 2 µl DNA and 1 µM primers. The oligonucleotide primers used to detect AIB1 were 5'-AGCCGATGTATCTTCTACAGG-3' and 5' AAAGCGGTCCTAAGGAGTCT-3'. The PCR reaction conditions were 10 minutes at 95°C followed by 50 cycles of 30 seconds each at 95°C, 58°C, and 72°C. Fluorescent data were collected during the 72°C step. A dilution series of the plasmid pcDNA3-AIB1 was used as a standard.

Acknowledgements

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Figure Legends

Figure 1 Effect of estradiol on AIB1 mRNA levels. MCF-7 cells, grown in full serum, were treated with media containing 1% CCS for 48 hrs followed by 10^{-7} M 17β-estradiol. The cytoplasmic RNA was harvested over 48 h. Following Northern blot analysis, AIB1 levels were analyzed by phosphorimager and corrected by GAPDH. Untreated cells (-48 h) were arbitrarily set at 100% and cells treated with hormone in serum free medium were analyzed in reference to this. Results are based on 3 separate experiments performed in duplicate; error bars represent SEM. Statistical analyses were performed using a *t*-test. *, $P<.05$ compared to untreated cells.

Figure 2 Effect of 17β-estradiol and antiestrogens on AIB1 mRNA levels. MCF-7 cells, grown in full serum, were treated with serum-free media plus **(a)** 10^{-7} M 17β-estradiol **(b)** 10^{-8} M ICI 182,780 or **(c)** 10^{-6} M OHT. The cytoplasmic RNA was harvested over 48 h. Following Northern blot analysis, AIB1 levels were analyzed by phosphorimager and corrected by GAPDH. Cells shifted from full serum to serum-free medium (0 h) were arbitrarily set at 100% and cells treated with ligands in serum-free medium were analyzed in reference to this. Insets represent a typical Northern blot. Results are based on 3 separate experiments performed in duplicate; error bars represent SEM. Statistical analyses were performed using a *t*-test. **(a)** No significance was found between the data points. **(b, c)** *, $P<0.05$ compared to untreated cells. **(d)** MCF-7 cells were treated with 10^{-8} M ICI 182,780 for 24 h, washed 2 times with IMEM followed by treatment with 10^{-7} M estradiol. Cytoplasmic RNA was harvested over 48 h. Following Northern blot analysis, AIB1 levels were analyzed by phosphorimager and corrected by GAPDH. Untreated cells (-24 h) were arbitrarily set at 100% and treated cells were analyzed in reference to this. Results are based on at least 3 separate experiments; error bars represent SEM. Statistical analyses were performed using a *t*-test. *, $P<0.05$ compared to untreated cells.

Figure 3 Estrogen and antiestrogen induced changes in AIB1 protein expression. **(a)** MCF-7 cells were treated with 1% CCS for 48 h, washed 2 times with IMEM followed by treatment with 10^{-7} M 17β-estradiol. Cell lysates were harvested over 48 h. and analyzed by Western blot with an anti-AIB1 antibody, stripped and reprobed with anti-actin antibody as a loading control (relative fold changes are shown numerically above lanes). Protein levels were analyzed by densitometry. **(b)** MCF-7 cells were treated with 10^{-8} M ICI 182,780 for 24, 48, and 72 h. Cell lysates were harvested and subjected to Western blot analysis using an anti-AIB1 antibody and staining with Ponceau S was used as a loading control (relative fold changes are shown numerically above lanes).

Figure 4 All-trans retinoic acid increases AIB1 gene expression and is reversed with estradiol. **(a)** MCF-7 cells were treated with 10^{-6} M atRA and the cytoplasmic RNA was harvested over 48 h. Following Northern blot analysis, AIB1 levels were analyzed by phosphorimager and corrected by GAPDH. Untreated cells (0 h) were arbitrarily set at 100% and treated cells were analyzed in reference to this. Results are based on 5 separate experiments; error represent SEM. Statistical analyses were performed using a t-test. *, $P<0.05$ compared to untreated cells. Inset represents a typical Northern blot. **(b)** MCF-7 cells were treated with 10^{-6} M atRA for 24, 48, and 72 h. Cell lysates were harvested and subjected to Western blot analysis using an anti-AIB1 antibody. Ponceau S staining was used as a loading control. Relative fold changes in AIB1 are indicated numerically above lanes. **(c)** MCF-7 cells were treated with 10^{-6} M atRA for 24 h, washed 2 times with IMEM followed by treatment with 10^{-7} M 17β-estradiol and the cytoplasmic RNA was harvested over 48 h. Following Northern blot analysis, AIB1 levels were analyzed by phosphorimager and corrected by GAPDH. Untreated cells (-24 h) were arbitrarily set at 100% and treated cells were analyzed in reference to this. Results are based on at least 3 separate experiments; error bars represent SEM. Statistical analyses were performed using a t-test. *, $P<0.05$ compared to untreated cells.
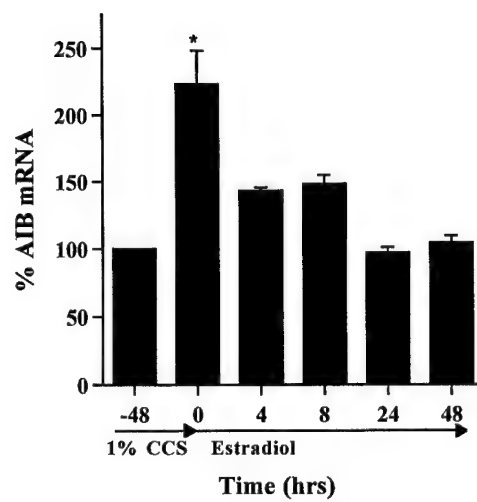
Figure 5 The half-life of AIB1 mRNA is unaffected by treatment with ICI, atRA, or estradiol. MCF-7 cells were treated with 5 μg/ml actinomycin D (act D) alone and in the presence of 10^{-8} M ICI, 10^{-6} M atRA, or 10^{-7} M estradiol and the cytoplasmic RNA was harvested over 16 h. Following Northern blot analysis, AIB1 levels were analyzed by phosphorimager and corrected by GAPDH. Untreated cells at 0 h were arbitrarily set at 100% and treated cells were analyzed in reference to this. Results are based on 2 separate experiments performed in duplicate.

Figure 6 ChIP assay demonstrates increased transcription following stimulation by ICI. MCF-7 cells were treated for 48 h with 10^{-7} M 17β-estradiol in the absence of serum. Cells were washed 2 times with IMEM and stimulated for 2 h with 10^{-8} M ICI. An antibody to RNA Pol II was used for immunoprecipitation and a fragment of AIB1 exon 4 was amplified and quantified by real time PCR. The amount of AIB1 DNA pulled down by IP was corrected for the amount of DNA in the input. The no antibody control indicates background levels of AIB1 pulled down non-specifically during IP and was arbitrarily set at 1. The amount of AIB1 DNA pulled down in the estradiol and ICI treated cells is expressed as fold induction over the background no antibody control. Results are based on at least 3 separate experiments; error bars represent SEM. Statistical analyses were performed using a *t*-test. *, $P<0.05$ compared to untreated cells.
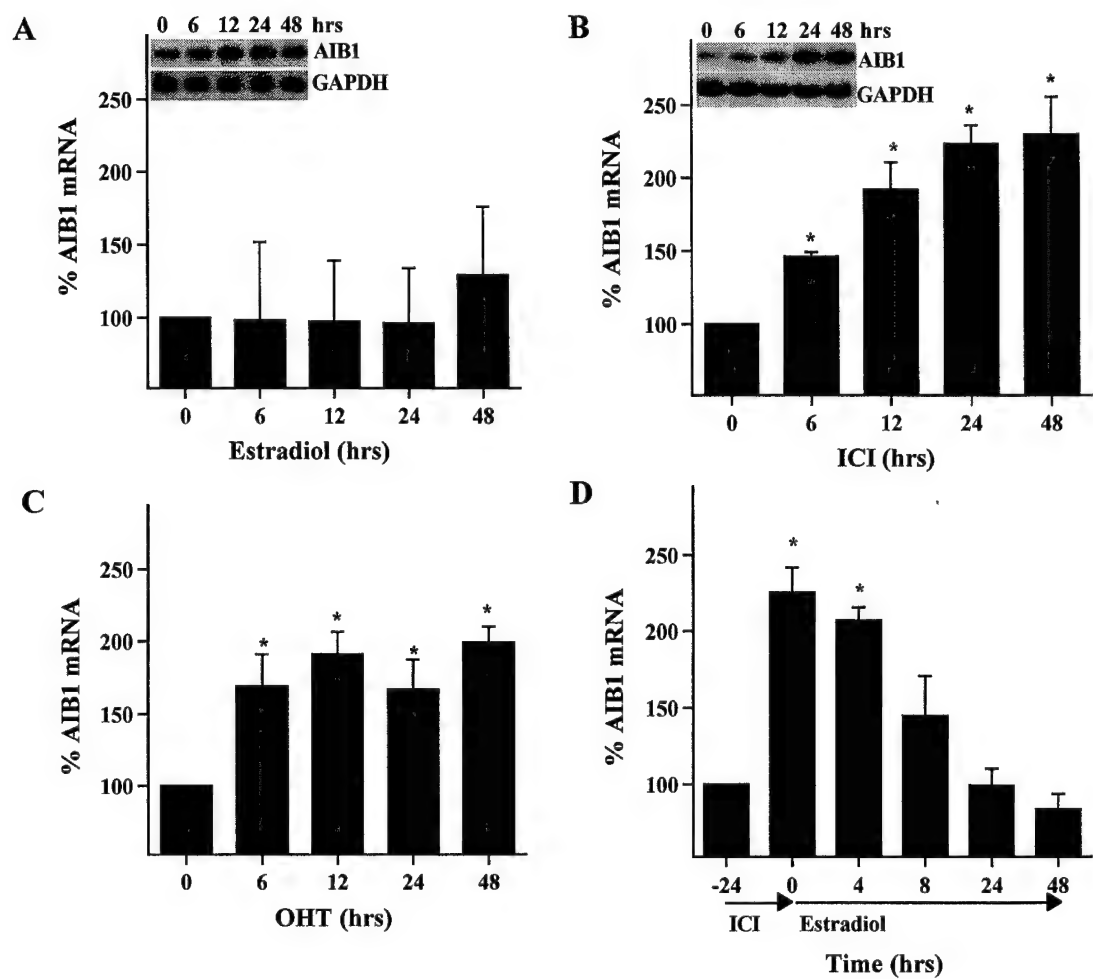
Figure 7 *De novo* protein synthesis is necessary for the induction of AIB1 mRNA by ICI and atRA. MCF-7 cells were treated with 10 µg/ml cycloheximide (CHX) alone and in the presence of 10^{-8} M ICI or 10^{-6} M atRA and the cytoplasmic RNA was harvested after 24 h. Following Northern blot analysis, AIB1 levels were analyzed by phosphorimager and corrected by GAPDH. Untreated control cells (C) were arbitrarily set at 100% and treated cells were analyzed in reference to this. Results are based on at least 3 separate experiments; error bars represent SEM. Statistical analyses were performed using a *t*-test. *, $P<0.05$ compared to untreated cells.

Figure 8 ICI and atRA act through TGF-β to increase AIB1 mRNA levels. **(a)** MCF-7 cells were treated with 10 ng/ml TGF-β and the cytoplasmic RNA was harvested over 48 h. Following Northern blot analysis, AIB1 levels were analyzed by phosphorimager and corrected by GAPDH. Untreated cells (0 h) were arbitrarily set at 100% and treated cells were analyzed in reference to this. Results are based on at least 3 separate experiments. Statistical analyses were performed using a t-test. *, P<0.05 compared to untreated cells. **(b)** MCF-7 cells were treated with 10^{-8} M ICI or 10^{-6} M atRA for 2 h in serum-free media, then 50 μg/ml anti-TGF-β was added to the media and the cytoplasmic RNA was harvested 24 h later. Following Northern blot analysis, AIB1 levels were analyzed by phosphorimager and corrected by GAPDH. Untreated cells (control - 0 h) were arbitrarily set at 100% and treated cells were analyzed in reference to this. Results are based on one experiment performed in triplicate. Statistical analyses were performed using a t-test. *, P<0.05 compared to untreated cells.

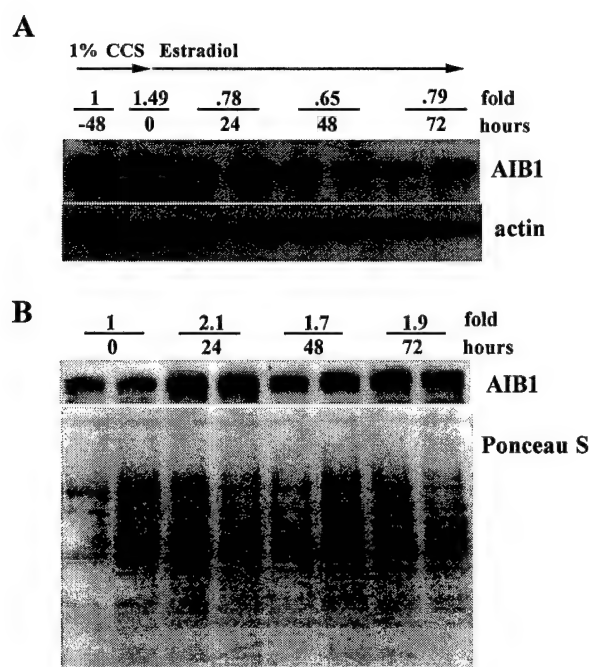**Table 1** Induction of AIB1 is not a result of antiproliferative events in MCF-7 cells. MCF-7 cells were treated with 10^{-8} M ICI, 10^{-6} M OHT, and 10^{-6} M atRA and harvested for cell cycle analysis over 48 h. Analysis was done by flow cytometry (Materials and Methods). Data represent the mean of 3 separate experiments.
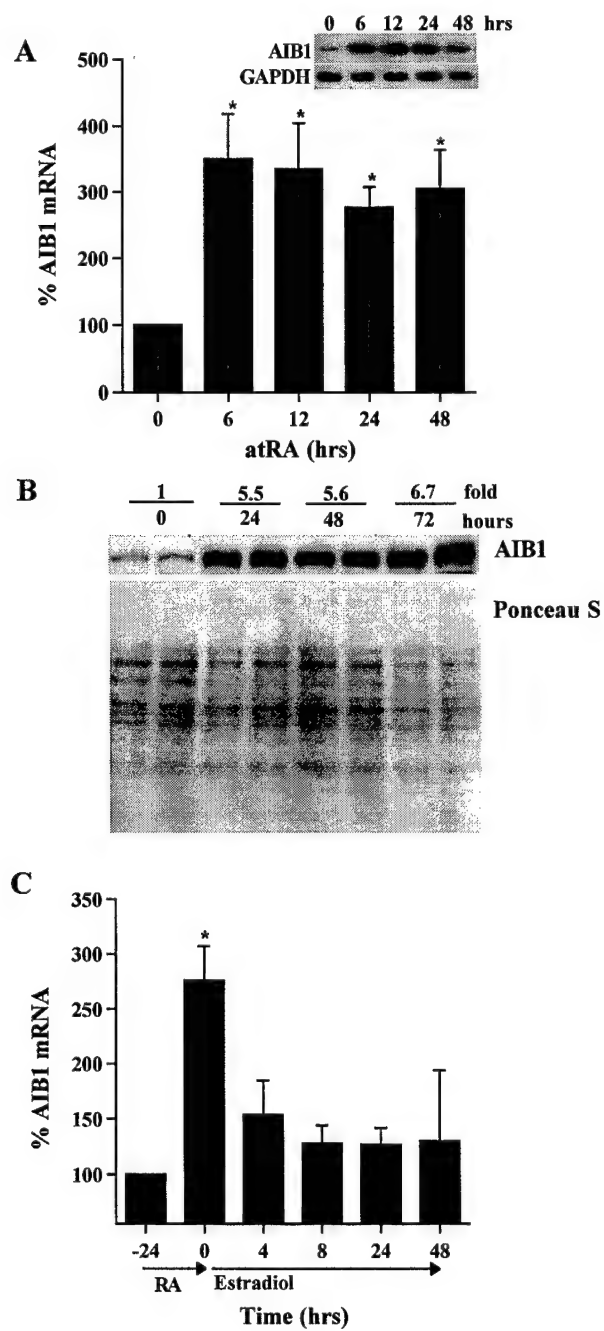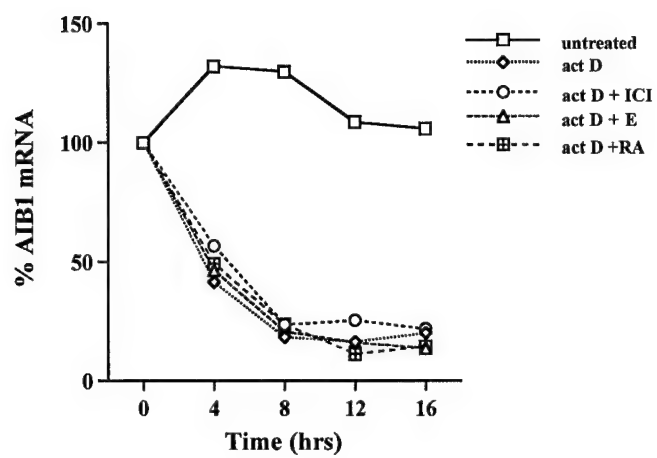
Lauritsen et al. Fig. 1
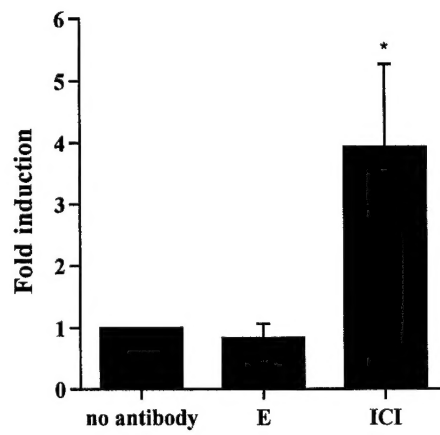
Lauritsen et al. Fig. 2

A

1% CCS Estradiol

$\underline{1}$	$\underline{1.49}$	$\underline{.78}$	$\underline{.65}$	$\underline{.79}$	fold
-48	0	24	48	72	hours

AIB1

actin

B

$\underline{1}$	$\underline{2.1}$	$\underline{1.7}$	$\underline{1.9}$	fold
0	24	48	72	hours

AIB1

Ponceau S

Lauritsen et al. Figure 3

Lauritsen et al. Fig. 4

Lauritsen et al. Fig. 5

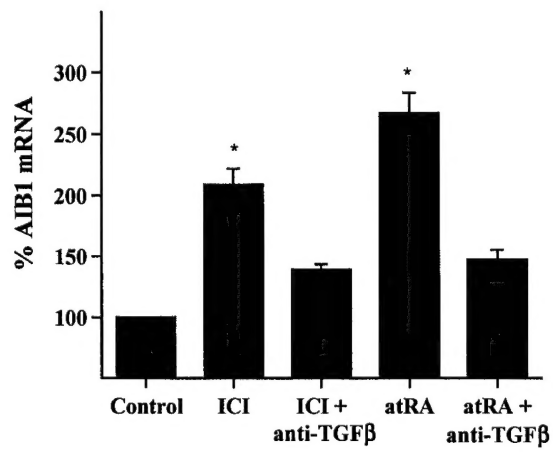**Lauritsen et al. Fig. 6**

Lauritsen et al. Fig. 7

A

% AIB1 mRNA (y-axis)

TGF-β (hrs)

0   6   12   24   48

B

% AIB1 mRNA (y-axis)

Control   ICI   ICI + anti-TGFβ   atRA   atRA + anti-TGFβ
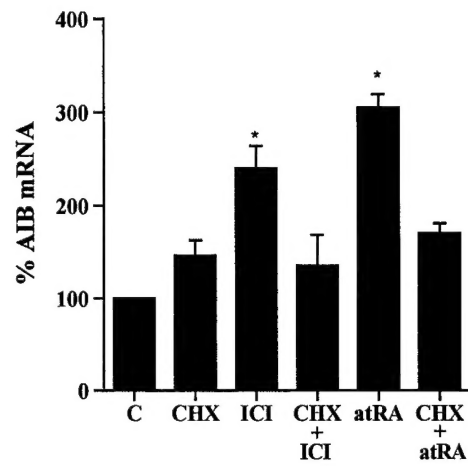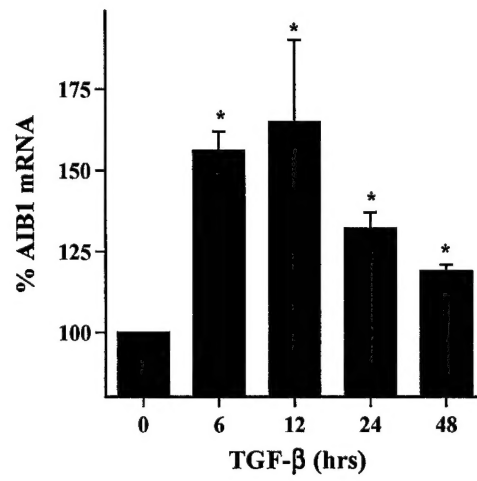
Lauritsen et al. Fig. 8

Cell cycle phase	ICI		OHT		atRA	
	% S	% G0/G1	% S	% G0/G1	% S	% G0/G1
Time (hrs) 0	45	45	52	27	46	47
6	45	41	37	41	50	37
12	27	57	25	58	35	44
24	6	88	11	76	29	60
48	6	89	23	64	17	72